PATENT
3645-0104P

IN THE U.S. PATENT AND TRADEMARK OFFICE

Applicant: Richard B. THOMPSON et al. Conf.: 9931

Appl. No.: 09/942,708 Group: 1651

Filed: August 31, 2001 Examiner: SRIVASTAVA, K.C.

For: DETERMINATION OF METAL IONS IN SOLUTION BY PHOTOLUMINESCENCE ANISOTROPY

DECLARATION UNDER 37 C.F.R. § 1.132

Honorable Commissioner of Patents
Washington, D.C. 20231

Sir:

I, Dr. Richard B. Thompson, hereby declare as follows:

1. I am a U.S. citizen, residing at 7106 Bristol Road, Baltimore, Maryland 21212.

2. I am presently employed as Associate Professor in the Department of Biochemistry and Molecular Biology of the University of Maryland School of Medicine, Baltimore. A copy of my Curriculum Vitae is attached.

3. I am a co-inventor of the subject matter of the above-identified U.S. Patent application. I am familiar with the specification and pending claims, and with the prosecution history of the application.

4. The Examiner has rejected claims 2, 5 and 13 of the

application as being obvious in view of U.S. Patent 5,545,517 to Thompson et al. and Thompson et al., *Journal of Fluorescence* (1992), in view of Toyo'oka et al., *Anal. Chem.* (1984). The Examiner asserts that the Thompson references describe a composition comprising apo-carbonic anhydrase and a dansylamide fluorophore. Toyo'oka is cited for the proposition that it would be obvious to substitute the 4-(2-hyrdroxyethylthio)-7-aminosulfonyl-2,1,3-benzoxadiazole ("ABD-M") fluorophore recited in claim 2 for the dansylamide described by Thompson et al. The Examiner asserts such substitution is obvious because ABD-M, "facilitates clear detection, does not give an interfering background fluorescence, has a long fluorescence wavelength so that interfering endogenous fluorescent materials in biological samples can be avoided but rather it fluoresces specifically only when combined with an analyte, i.e. (human) apo-carbonic anhydrase."

5. The use of ABD-M in combination with apo-carbonic anhydrase ("apo-CA") provides results, as described below, that would not have been expected by the practitioner of ordinary skill in the art of fluorescence spectroscopy at the time the present invention was made who would read the Thompson references and Toyo'oka et al. These unexpected results are evidence of the unobviousness of the invention described in the present claims 2, 5 and 13.

6. In particular (1) ABD-M binds more tightly to holo-CA than dansylamide. This is shown in the attached Exhibit 1, a Table excerpted from C.A. Fierke and R. B. Thompson, "Fluorescence-based biosensing of zinc using carbonic anhydrase," *BioMetals* **14**, 205 - 222 (2001). There was simply no way to predict the binding constant of the interaction of ABD-M with holo-CA at the time the present invention was made. Neither the Thompson references nor the Toyo'oka paper provided this information.

7. ABD-M not only fluoresces when bound by carbonic anhydrase (CA), but its quantum yield increases about three-fold. This is also shown in the attached Exhibit 1. This result was unexpected because binding of fluorophores to proteins is frequently accompanied by partial or complete quenching of the fluorescence; if its fluorescence is completely quenched upon binding, a fluorescent aryl sulfonamide is unworkable in an anisotropy assay as in the present application. Examples of quenching of a fluorophore by binding to a protein include anti-fluorescein antibody binding of fluorescein (see the work of E. W. Voss, et al. for example, R. M. Watt and E. W. Voss, "Mechanism of quenching of fluorescein by anti-fluorescein IgG antibodies" *Immunochemistry* **14**, 533 (1977)), and binding of flavins to many proteins, such as bacterial luciferase. In many cases the quenching is due to electron transfer from tryptophan,

a constituent of nearly all proteins and indeed of apo-CA.

8. ABD-M's emission spectrum is significantly blue-shifted upon binding to CA compared to the emission spectrum of ABD-M free in solution. This is shown in the attached Exhibit 2, figures showing the emission spectra of ABD-M and dansylamide bound to apo-CA. While it is true that fluorophores that exhibit substantial blue shifts upon binding to proteins are not unknown, such fall into only a few structural classes, notably the anilinonaphthalene sulfonate derivatives (to which dansylamide is similar). By far the majority of fluorophores which bind to proteins (either noncovalently or covalently) exhibit little change in their emission wavelength, if they emit at all (see above). That the blue shift upon ABD-M's binding was substantial enough to permit a broadened dynamic range of measurement of divalent metal ion concentration was unexpected.

9. The above unexpected properties of the association of ABD-M with holo-carbonic anhydrase provide certain advantages to the use of apo-carbonic anhydrase with ABD-M (and other aryl sulfonamides recited in the pending claim 2) in fluorescence spectroscopic techniques, especially in the method described in the present specification. Tighter binding of the aryl sulfonamide to apo-CA is advantageous because a lower concentration of sulfonamide may be used and thus the emission of

the bound sulfonamide represents a greater fraction of the total emission, and consequently one can detect a lower concentration of holo-CA, and thus of zinc or other divalent metal cation in a sample. A higher quantum yield allows use of smaller samples overall and/or an assay that is more sensitive to smaller concentrations of analyte. The substantial blue-shift in the emission spectrum of ABD-M upon binding to apo-CA provides an unexpected broadened dynamic range for measurements of interaction of the complex with divalent metal cation analytes.

The increase in dynamic range of measurement is shown in the attached Exhibit 3, which shows titrations of the amount of zinc ion in solution measured by the anisotropy method of the instant invention using ABD-M at varying emission wavelengths: by merely changing the emission filter one can accurately measure the concentration of free Zn over a range of approximately 4 orders of magnitude, instead of only two orders. Dansylamide does not offer such a dynamic range in a steady state measurement at all, and only if more costly and complex lifetime techniques are used (*see,* R. Thompson and M. W. Patchan, *J. Fluor* 5, 123 (1995), of record).

It should be noted that although dansylamide also binds to holocarbonic anhydrase in a zinc-dependent manner (as disclosed in US Pat. No. 5,545,517) and exhibits a blue shift and quantum yield increase upon binding (as determined by R. F. Chen and J. C.

Kernohan, *J. Biol. Chem.* **242** 5813 - 5823 (1967), referenced in U.S. '517 and copy attached), it is almost unworkable in the divalent cation quantitation methods of the present invention. This is because its average fluorescent lifetime in the bound form (also determined by Chen and Kernohan) increases more than seven-fold upon binding. As the Perrin equation (which is well known in the art) shows, the measured steady state anisotropy is primarily a function of the ratio of the lifetime to the rotational correlation time of the emitting component(s). The essence of the invention is to increase anisotropy in a zinc-dependent manner by increasing the rotational correlation time of the photoluminescent aryl sulfonamide several-fold by having it bind to the more massive protein; if the lifetime also increases upon binding, the increase in anisotropy is thereby much reduced or eliminated, providing a much smaller signal.

On the other hand, ABD-M unexpectedly retains a sufficiently short lifetime so as to be useable in the anisotropy methods of the present invention. Such a result would not be expected by the person of ordinary skill in the art who reads the Thompson references for their teachings of the properties and utility of dansylamide. The fluorescent lifetime of ABD-M when bound to a protein is not available from the Toyo'oka reference, and so the artisan of ordinary skill is not motivated to make the present invention based upon the teachings of U.S. Patent 5,545,517, Thompson et al., *Journal of Fluorescence* (1992) and Toyo'oka et

al., *Anal. Chem.* (1984).

10. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 10/1/04

By _____ 10/1/04
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Postdoctoral Fellow, University of Maryland, School of Medicine, 1981-1984

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RESEARCH EXPERIENCE:

1990-present: University of Maryland School of Medicine. Associate Professor with emphasis on fiber optic biosensors and fluorescence spectroscopy.

1984-1990 - Naval Research Laboratory, Washington, DC. Developing ultrasensitive fluorescence immunoassay systems and fiber optic sensors.

1981-1984 - University of Maryland, School of Medicine. Fluorescence and High Pressure Work with Professor Joseph R. Lakowicz.

1975-1981 - University of Illinois. Determined Primary Structure of Beta Subunit of Bacterial Luciferase, with Professor Thomas O. Baldwin. Thesis title, "Amino Acid Sequence Studies on the Beta Subunit of Bacterial Luciferase".

1974-1975 - Northwestern University. Determined Primary Structure of Hippopotamus Cytochrome c, with Professor E. Margoliash.

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INVITED LECTURES:

R. B. Thompson, "Fluorescence-based biosensing and imaging of free metal ions in complex matrices," Second Gordon Conference on Environmental Bioinorganic Chemistry, Bates College, Lewiston, ME, June 2004

R. Bozym, H. H. Zeng, R. Rosenthal, G. Fiskum, and R. B. Thompson, "Quantiating subnanomolar free zinc *in vivo* and *in vitro* with ratiometric fluorescence biosensors," Fifth International Zinc Signals Conference, Aarhus, Denmark, June 2004.

R. B. Thompson, "Advanced fluorescence-based biosensors for free Zn measurments *in vitro* and *in vivo*," Department of Biochemistry and Molecular Biology, University of Maryland School of Medicine, Baltimore, MD, May, 2004

R. B. Thompson, "*In vivo* and intracellular sensing and imaging of free zinc ion using a fluorescence-based biosensor," SPIE Conference on Advanced Clinical and Biomedical Diagnostic Systems, Photonics West, San Jose, CA, January, 2004.

R. B. Thompson, "Free Zn ind Cu in the brain: Elucidating their biology and role in disease using fluorescence-based biosensors," Fourth International Zinc Signals Conference, Grand Cayman Island, B.W. I. May, 2003

R. B. Thompson and C. A. Fierke, "Carbonic anhydrase-based metal ion biosensors," Fifth Coolfont ONR Biomimetics Program Meeting, Coolfont, WV, April, 2003.

R. B. Thompson, "Zinc and neurodegeneration: Measurements using novel fluorescence-based biosensors," Department of Anesthesiology and the Neuroprotection Focus Group of the Program in Neuroscience, University of Maryland School of Medicine, Baltimore, MD, April, 2003

"Fluorescence-based biosensors of metal ions at low concentrations *in vitro*," Cheasapeake Biological Laboratory, University of Maryland Center for Environmental Science, Solomons, MD, September, 2002;

30th Annual Meeting of the American Society for Photobiology, Quebec City, Canada, July 2002; "Fluorescence-based biosensors of metal ions at low concentrations in vitro, "

Zinc Signals 2002, Grand Cayman Island, B. W. I., April, 2002; "Novel ratiometric approaches to intracellular zinc sensing,"

Fourteenth Annual Meeting of the IEEE Lasers and Electro-Optics Society, San Diego, CA, November, 2001, "Quantitative fluorescence-based biosensing and imaging of zinc and copper in complex media,".

University of Kentucky Medical Center, Lexington, KY, November, 2001; "Fluorescence-based fiber optic biosensors for metal ions in complex media,"

Fourth Coolfont Symposium on Biomolecular Recognition, Berkeley Springs, WV, November, 2000, "Fluorescence-based metal ion biosensors,".

Second ONR/EPA Workshop on Chemistry, Toxicity, and Bioavailability of Copper and its Relationship to Regulation in the Marine Environment, Annapolis, MD, November, 2000, "Fluorescence-based biosensing of Cu(II) in Sea Water,".
27th Annual Conference of the Federation of Analytical Chemistry and Spectroscopy Societies, Nashville, TN, September, 2000."Fluorescence-based metal ion biosensing: Application to imaging zinc release in the hippocampus,"

Woods Hole Oceanographic Institute, Woods Hole, MA, July 2000, "Fluorescence-based metal ion biosensing in complex media,".

Center for Photochemistry, Bowling Green State University, Bowling Green, OH, April, 2000, "Determination of metal ions by fluorescence anisotropy,".

Department of Biochemistry, University of Michigan, Ann Arbor, MI, April, 2000, "Determination of metal ions by fluorescence anisotropy."

Department of Chemistry and Biochemistry, University of Delaware, Newark, DE, April, 2000, "Determination of metal ions by fluorescence anistropy,".

Spring Hippocampal Research Conference, Grand Cayman Island, B. W. I., April 2000, "Imaging Zn release from the hippocampus by fluorescence microscopy using a biosensing approach,".

SPIE Conference on In-vitro Diagnostic Instrumentation, San Jose, CA, January, 2000, "Issues in enzyme-based metal ion biosensing in complex media," .

SPIE Conference on Advanced Materials and Optical Systems for Chemical and Biological Detection, Boston, MA, November, 1999, "Improved response of a fluorescence-based metal ion biosensor using engineered carbonic anhydrase variants,".

SPIE Conference on Advances in Fluorescence Sensing Technology IV, San Jose, CA, February, 1999, "Reagentless biosensing of transition metals by fluorescence anisotropy."

25th Annual Conference of the Federation of Analytical Chemistry and Spectroscopy Societies, Austin, TX, October, 1998, "Biosensing free Cu, Zn, Co, Cd, and Ni at ppb levels in solution by fluorescence anisotropy."

31st Midwestern Regional ACS Meeting, Symposium on Recent Advances in Fluorescence Spectroscopy, Milwaukee, WI, June, 1998: "Determination of transition metal ions at picomolar levels free in solution with fluorescence polarization."

Department of Chemistry and Biochemistry, University of Massachusetts, Dartmouth, May, 1998: "Determination of transition metal ions at picomolar levels by fluorescence polarization."

1998 Spring International Hippocampal Research Conference, Grand Cayman Island, British W.I., April, 1998: "New technology for visualizing zinc levels in the brain."

Department of Biochemistry, Duke University Medical Center, Durham, NC, February 1998: "Determination of metal ions by

fluorescence anisotropy,"

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD, October, 1997: Determination of metal ions by fluorescence anisotropy"

MicroFab Technologies, Inc., Plano, TX, October 1997: Determination of Zn(II) by fluorescence anisotropy"

RAND Corporation, Washington, DC, June 1997: "Recent developments in optical biosensors."

Department of Biochemistry and Molecular Biology, University of Maryland School of Medicine, March, 1997: "Fluorescence-based fiber optic metal ion biosensors."

Second Biennial ONR Coolfont Conference on Molecular Recognition, Berkeley Springs, WV, November, 1996: Fiber optic metal ion biosensors."

U.S. Department of Agriculture, Center for Devices and Radiological Health, Rockville, MD, June, 1996, "Fluorescence-based fiber optic sensors."

Fifth Biennial DoD Photonics Conference, McLean, VA, March, 1996, "Fluorescence-based fiber optic chemical sensors."

University of Pennsylvania, Department of Chemistry, Philadelphia, PA, February, 1996: "Fluorescence-based fiber optic biosensors."

University of Maryland School of Pharmacy, Baltimore, MD, October, 1995: "Fluorescence-based fiber optic biosensors."

International Conference on Carbonic Anhydrases, Oxford, England, July, 1995: Carbonic Anhydrase as a Biosensor Transducer."

Second European Science Foundation Artificial Biosensing Interfaces Workshop, Tampere, Finland, June, 1995: "Metal Ion Biosensors Based on Fluorescence Energy Transfer."

Conference on Chemical, Biochemical, and Environmental Fibre Sensors VII, Munich, Germany, June, 1995: "Fluorescence Energy Transfer-Based Fibre Optic Biosensors."

Conference on Lasers and Electro-Optics/Quantum Electronics and Laser Spectroscopy '95, May, 1995, Baltimore, MD: "Fiber Optic Metal Ion Biosensors Based on Fluorescence Resonance Energy Transfer."

ONR Conference on Molecular Recognition, Coolfont Resort, Berkeley Springs, WV, October, 1994: "A Fluorescence-Based Fiber Optic Metal Ion Biosensor."

Gordon Research Conference on Bio/Analytical Sensors, Colby Sawyer College, New London, NH, July, 1994: Energy Transfer-Based Fiber Optic Metal Ion Biosensors."

Gordon Research Conference on Lasers in Medicine and Biology, Kimball Union Academy, Meriden, NH, July, 1994: "Fluorescence Lifetime-Based Fiber Optic Chemical Sensors."

University of Utah, Salt Lake City, UT, June, 1994: "Fluorescence Lifetime-Based Fiber Optic Biosensors".

University of Maryland Baltimore County, December 1993: "Fiber Optic Chemical Sensors".

Third International Conference on Methods and Applications in Fluorescence Spectroscopy, Prague, Czech Republic, October 1993: "Fluorescence-Based Fiber Optic Sensors."

AAAS Conference Science Innovation '93, Boston, MA, August, 1993: "Fluorescence Lifetime-Based Fiber Optic Sensors."

Fourth Gordon Conference on Bioanalytical Sensors, Ventura, CA, March, 1993: "Phase Fluorimetric Fiber Optic Chemical Sensors."

Wellman Laboratory of Photomedicine, Harvard Medical School, Boston, MA, March, 1993: "Fiber Optic Chemical Sensors Based on Phase Fluorometry."

Annual Meeting of the IEEE Lasers and Electro-Optics Society, Boston, MA, November, 1992: "A Fiber Optic pH Sensor Based on Phase Fluorimetric Lifetime Measurement."

Department of Biomedical Engineering, Johns Hopkins University Medical School, June 1992: "Fiber Optic Biosensors."

Thirty-Sixth Annual Meeting of the Biophysical Society, Houston, TX, February, 1992; "Applications of Fluorescence-Based Fiber Optic Sensors."

Fourth Annual Meeting of the IEEE Lasers and Electro-Optics Society, San Jose, CA, November, 1991; "Fiber Optic Biosensors."

International Symposium on Innovative Fluorescence Methodologies in Biochemistry and Medicine, Frascati, Italy, September, 1991; Fluorescence Methodologies as Applied to Fiber Optic Sensors for Immunology and for Diagnostics."

Georgetown University School of Medicine, Washington, DC, April 1990, "Fiber Optic Biosensors."

Second Gordon Research Conference on Bioanalytical Sensors, Ventura, CA, March, 1990; "Improving the Sensitivity of Waveguide-Binding Fiber Optic Biosensors."

Eleventh International Meeting of the IEEE Engineering in Medicine and Biology Society, Seattle, WA, November, 1989; "Waveguide Parameter for Waveguide-Binding Fiber Optic Biosensors."

Chemical Society of Washington, Washington, DC, October, 1989; "Biosensor Fundamentals."

Beckman Laser Institute, Irvine, CA, February, 1989; "Fiber Optic Biosensors."

City of Hope National Medical Center, Duarte, CA, February 1989; "Fiber Optic Biosensors."

Allied/Instrumentation Laboratories, Andover, MA, September 1985; "Ultrasensitive Fluorescence Immunoassays Employing Vesicle Lysis."

Amoco Research Center, Naperville, IL, November, 1985; "Ultrasensitive Fluorescence Immunoassays Based on Vesicle Lysis."

Cardiovascular Research Institute, University of California at San Francisco, March 1984; "Effects of High Pressure on the

Self-Association of Melittin."

Naval Research Laboratory, Washington, DC, January 1984; "Fluorescence Studies of Biomolecular Structure Under High Pressure."

Stanford University, May 1981; "Amino Acid Sequence Studies on Bacterial Luciferase."

AWARDS AND PROFESSIONAL ACTIVITIES:

ad hoc Member, Science Policy Committee of FASEB, 1999-2000

Member, Editorial Board of *Journal of Biomedical Optics*, 1997-present

Member, Editorial Board of *Journal of Fluorescence*, 1994-7; 2001 - present
Member, Editorial Board of *Journal of Biochemical and Biophysical Methods*, 1996-2003

Member (*ad hoc*), NIH Study Section on Bioanalytical Engineering and Chemistry, 2004

Member, NSF SBIR/STTR Panel on Biotechnology Sensors, 2004

Member, NSF Panel on Biophotonics, 2000-

Member, Department of Energy NABIR Review Panel, 1997

Member, NIH Study Section on SBIR/STTR for National Center for Research Resources, NIH, 1996, 1997.

Member, NSF SBIR Review Panel: Earth Sciences, 1995 - 1998

Co-Chair, SPIE Conference on Ultrasensitive Clinical Diagnostics, San Jose, CA, 1996- present.

Co-Chair, SPIE Conferences on Advances in Fluorescence Sensing, 1993, 1995, 1997, 1999, 2001.

Nominee of University of Maryland System to be Searle Scholar, November, 1993.

Member, Organizing Committee for the Fourth International Conference on Methods and Applications in Fluorescence Spectroscopy, Cambridge, England, 1995.

Member, ONR Site Visit Team for Biotechnology Institutes in Russia, June 1993.

Member, ASEE Evaluation Board for Office of Naval Technology Fellowships, December, 1992.

Member, IEEE Lasers and Electro-Optics Society Annual Meeting Program Committee, January, 1992.

Awarded U.S. Navy Special Act Award for Exceptional Performance in Support of Operation Desert Storm, June 1991.

Member, National Research Council Panel on New Measurement Technologies for the Oceans, 1990 - 1.

Awarded Promotion, December, 1989.

Received Outstanding Performance Rating, July 1989.

Awarded Quality Salary Increase, January 1988.

Received Chemistry Division Award for Superior Technical Publication, February 1987.

Awarded National Research Council Associateship, April 1984.

PROFESSIONAL SOCIETIES:

Biophysical Society
American Society for Biochemistry and Molecular Biology
American Association for the Advancement of Science
American Chemical Society
U.S. Naval Institute
Society of Photooptical Instrumentation Engineers

CURRENT RESEARCH SUPPORT:

ACTIVE as of August 31, 2004:

NIH 1 RO1 EB 003924-01 08/15/04 - 5/31/08
NIH / NIBIB $1,584,000 total costs
"Zinc fluorescence biosensing and imaging technology,"

NSF OCE 0425564 09/01/04 - 08/31/07
National Science Foundation $ 624,000 total costs
"Collaborative Research: Determination of oceanic copper and zinc using biosensor technology,"

NOAA CICEET 09/01/04 - 8/31/06
Cooperative Institute for Coastal and Estuarine Environmental Technology $ 351,000
"Assessment of anthropogenic copper pollution in Waquoit Bay NERR,"

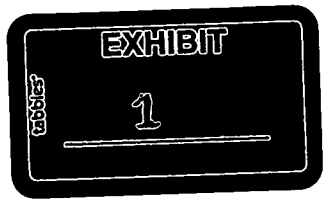

Table 2. Aryl sulfonamides used in zinc sensing.

Probe	Free/Bound	λ_{exc}, nm	λ_{em}, nm	QY, relative	$< \tau >$, nsec	K_D, μM	log ε	aniso	ratio	λ_1/λ_2	References
DNSA	Free	330	560	0.08	3		3.52		0.039	450/560	(Chen & Kernohan 1967; Thompson & Jones 1993)
	Bound		450	0.55	22.1	0.8			1.6		
ABD-N	Free	435	602	0.086	0.34		3.94	0.09	3	560/680	(Thompson *et al.* 2000a)
	Bound	420	558	1.0	4.98	0.9		0.23	7.5		
ABD-M	Free	390	528	0.33	0.8		3.9	0.03			(Thompson *et al.* 1998b)
	Bound	380	492	1.0	1.53	0.3		0.32			
Azosulfamide	Free	501				0.01	4.5				(Thompson & Patchan 1995b)
	Bound										
Dapoxyl sulfonamide	Free	365	615	0.01	0.22		4.3		3	535/685	(Thompson *et al.* 2000b)
	Bound		535	1.00	3.60	0.13			38		
BTCS	Free	466	504	0.88	2.53		4.56	0.05			(Thompson *et al.* 2000b)
	Bound			1.00	2.71			0.25			
Elbaum's	Free	495	505	1.0	4.0		4.9	0.01			(Elbaum *et al.* 1996)
	Bound			0.96		0.002		0.10			

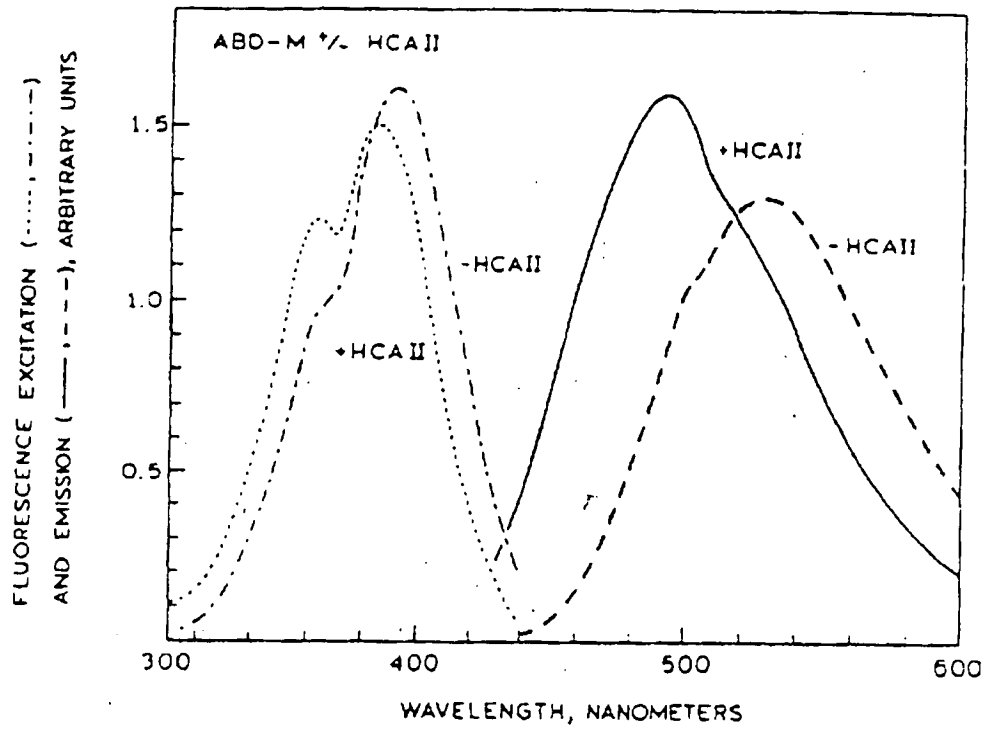

Figure 3. Excitation and emission spectra of ABD-M. Uncorrected excitation spectra are depicted for ABD-M in the absence (-·-·-, mission at 524 nm) and presence of holoenzyme (···, emission at 496 nm). Uncorrected emission spectra (excitation at 368 nm) are depicted for ABD-M bound to the holoenzyme (—) and free in solution (– – –).

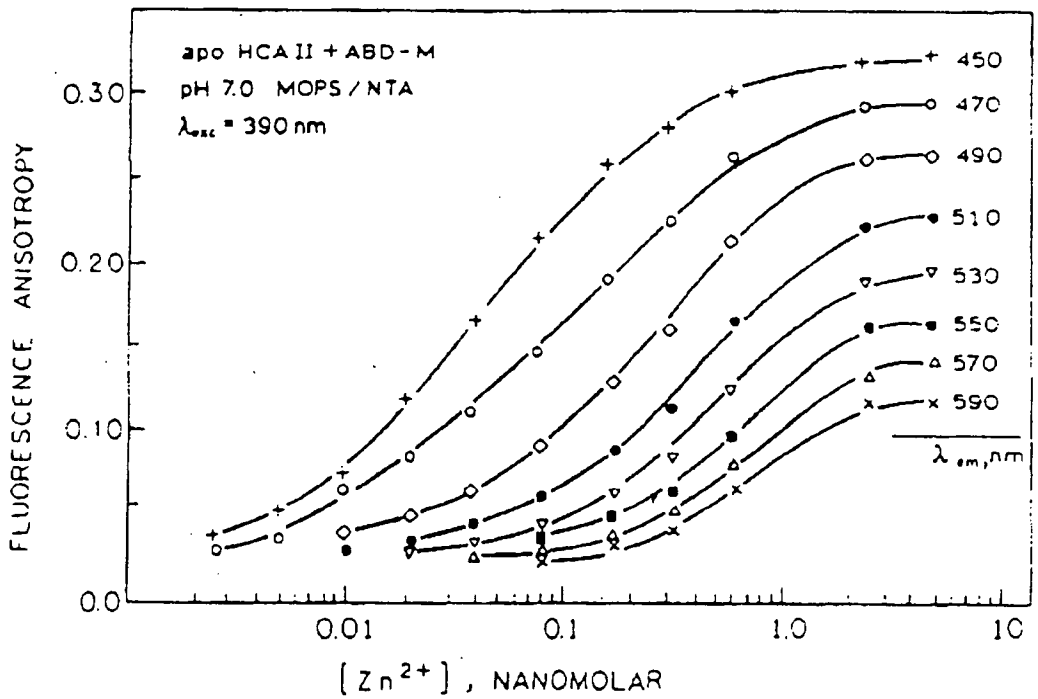

Figure 5. Fluorescence anisotropy of ABD-M plus apo-CA as a function of free Zn(II) concentration, measured at emission wavelengths of 450 nm (+), 470 nm (○), 490 nm (◇), 510 nm (●), 530 nm (▽), 550 nm (■), 570 nm (△), and 590 nm (×).

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 242, No. 24, Issue of December 25, pp. 5813-5823, 1967
Printed in U.S.A.

Combination of Bovine Carbonic Anhydrase with a Fluorescent Sulfonamide

(Received for publication, July 17, 1967)

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SUMMARY

Bovine erythrocyte carbonic anhydrase forms a highly fluorescent complex with 5-dimethylaminonaphthalene-1-sulfonamide (DNSA). The binding, studied either by enhancement of ligand fluorescence or by the quenching of protein ultraviolet fluorescence, shows that only 1 mole of DNSA is bound per mole of protein; the dissociation constant at pH 7.4 is 2.5×10^{-7} M. The fluorescence of free DNSA in water has peak emission at 580 mμ and a quantum yield of only 0.055, but bound DNSA has an emission maximum at 468 mμ and a yield of 0.84. Arguments are presented to explain the large emission blue shift on the basis that the binding site is extremely hydrophobic *and* that the $-SO_2NH_2$ group of the ligand loses a proton upon binding to the enzyme. The binding appears specifically to involve the sulfonamide site known to exist in carbonic anhydrase; several other "fluorescent probe" compounds showed no evidence of binding to the enzyme.

Calculation of the energy transfer efficiency gave the surprising result that 85% of the photons absorbed by the 7 tryptophan residues are transferred to the single bound DNSA molecule. The transfer efficiency is much higher than hitherto observed for a protein having only one 5-dimethylaminonaphthalene-1-sulfonyl group. Although the diameter of the protein is roughly 51 A, the bound DNSA group is probably within the critical transfer distance R_0 (≈ 21.3 A) of all the tryptophans. The effective average distance between DNSA and tryptophan was found to be 16 A. The fluorescence properties of the complex were quite different from those of a conjugate prepared by reaction of 5-dimethylaminonaphthalene-1-sulfonyl chloride with carbonic anhydrase. Various considerations lead to the conclusion that the sulfonamide-binding site and the tryptophan residues are in the interior of the protein.

The tryptophan fluorescence of the protein was 73% quenched by the binding of 1 DNSA molecule. Although large, this degree of quenching was less than the over-all efficiency of energy transfer of photons absorbed by the protein. This result indicates that the fluorescence efficiencies of the 7 tryptophans are different, and that DNSA is bound in such a way that energy transfer occurs with greater probability from those tryptophan residues which are relatively less fluorescent.

DNSA inhibits the esterase activity of carbonic anhydrase as tested with the substrate, *p*-nitrophenyl acetate. Direct measurements of fluorescence decay times permitted calculation of the rotational relaxation time of carbonic anhydrase from depolarization of fluorescence data. The value of about 30 nsec for the relaxation time is consistent with a low degree of molecular asymmetry.

In 1940, Mann and Keilin (1) reported the discovery that sulfonamides inhibit carbonic anhydrase. The reason for the highly specific and potent inhibition of this enzyme by this class of compounds is still not clear, although a number of facts bearing on the question is known. All sulfonamides which inhibit animal carbonic anhydrases have the $-SO_2NH_2$ group attached directly to an aromatic nucleus, which is either homocyclic or heterocyclic (2). The effectiveness of the inhibitor seems to be related to the ease with which a proton can dissociate from the sulfonamide group. There is evidence that such an ionization occurs before the inhibitor binds to the enzyme (3, 4). Inhibitory sulfonamides appear to be bound to the enzyme at a site which involves the metal ion at the active center (5, 6), and the sulfonamide-binding site may be identical with, or may overlap, the CO_2-binding site (7). However, sulfonamides do not form strong coordination bonds with transition metal ions such as Zn^{++}, and an interaction of the aromatic nucleus of the inhibitor with the protein must presumably make a large contribution to the observed stability of the complexes which are formed.

In this paper, we present some evidence for a hydrophobic binding site in bovine erythrocyte carbonic anhydrase B based on the properties of a highly fluorescent complex of the enzyme containing the sulfonamide, DNSA.[1] The results of fluorometric

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[1] The abbreviations used are: DNSA, 5-dimethylaminonaphthalene-1-sulfonamide; DNS-, 5-dimethylaminonaphthalene-1-sulfonyl-; CA(DNSA), a complex of carbonic anhydrase with

titrations show that only 1 DNSA molecule is bound by carbonic anhydrase. Emission spectral measurements show that the DNSA must be bound to a region of very low dielectric constant, and energy transfer calculations indicate that the sulfonamide may be in close association with some, if not most, of the tryptophan residues. In short, DNSA is a "fluorescent probe" of the sulfonamide-binding site of carbonic anhydrase. Several fluorescent dyes have been used as probes of hydrophobic binding sites (8–10). Typical of this class of dyes is 8-anilinonaphthalene-1-sulfonic acid, which is virtually nonfluorescent when free in aqueous solution but is reported to have a quantum yield of 0.75 when bound to bovine serum albumin (8). The spectra and quantum yields of such dyes are sensitive to conformational changes, which may affect the properties of the binding site (11–13). Recently, it was reported (14) that the DNS- derivatives of amino acids (so-called dansylamino acids) have properties similar to those of ANS and can be used as fluorescent probes; thus, it is not altogether surprising that DNSA can also fulfill this function. However, the binding of DNSA to carbonic anhydrase appears to involve those factors which render the inhibition by sulfonamides so specific and effective, and provides information not otherwise obtainable concerning interaction with this class of ligands. Neither ANS nor the DNS-amino acids, on the other hand, appear to bind to this enzyme.

EXPERIMENTAL PROCEDURE

Reagents—Electrophoretically pure bovine carbonic anhydrase B was used in all experiments, and was the same sample used in a previous study (4). Enzyme concentrations were estimated spectrophotometrically, with $\epsilon_{280} = 5.7 \times 10^4$ M^{-1} cm^{-1} (15). Bovine serum albumin was a crystalline sample obtained from Armour (Lot A69805) and defatted by charcoal treatment (16). DNSA was prepared according to Weber (17) and recrystallized from ethanol. Stock solutions containing 10^{-3} M DNSA were made by dissolving a weighed amount of the dried sulfonamide in 10^{-2} M HCl; these solutions were stable for at least 1 week. The extinction coefficient of DNSA in 60% (v/v) ethanol and the absorption spectra at various pH values matched reported values (17, 18). Purity of DNSA was also confirmed by thin layer chromatography in several solvent systems (19). ANS (Baker) was recrystallized as the magnesium salt (8). DNS- derivatives of glycine, L-proline, L-glutamic acid, and DL-tryptophan were purchased from British Drug Houses, and their purity was established by thin layer chromatography (19). Ethoxzolamide (6-ethoxybenzothiazole-2-sulfonamide) was a gift from Upjohn and was used without further purification. DNS-chloride was obtained from Baker and recrystallized from acetone before use.

Esterase Activity of Carbonic Anhydrase—The procedure of Armstrong, Myers, Verpoorte, and Edsall (20) was followed, except that the buffer was 0.02 M potassium phosphate, pH 7.4. The rate of nonenzymic hydrolysis of *p*-nitrophenyl acetate as followed in a 1-cm cell at 348 mμ was less than 0.005 optical density unit per min; such hydrolysis was corrected for according to Armstrong *et al.* (20).

Optical Measurements—Absorbance was measured in Beckman DU and Cary model 11 spectrophotometers. Fluorescence intensities and spectra were obtained with Aminco-Bowman spectrophotofluorometers. Cells of 1-cm path length were used for fluorometric titrations, but a microcell having internal cross-sectional dimensions of 0.29 × 0.29 cm was used for spectral measurements. Detailed description of the instrumentation, its use, the methods of calibration to give corrected excitation and emission spectra, and the calibration curves have been published (21, 22). Fluorescence quantum yields were obtained by comparing the area under the corrected emission spectrum with that of a standard[2] with a known quantum yield; the procedure was the same as that used in a previous study (23). Fluorescence polarization, P, was measured with the Aminco-Bowman apparatus with linearly polarized excitation (27). We calculated P from the relation (28)

$$P = \frac{I_{VV} - GI_{VH}}{I_{VV} + GI_{VH}}$$

where I is the observed fluorescence intensity and the first and second subscripts refer to the orientation of the polarizer and analyzer, respectively, and G is a correction factor for polarization introduced by the emission monochromator grating and is given by $G = I_{HV}/I_{HH}$.

Fluorescence decay time, τ, was measured with a TRW Instruments nanosecond flash apparatus, which was modified as previously described (29).

Unless otherwise specified, all experiments were conducted in 0.02 M phosphate buffer, pH 7.4, at 24° ± 1°.

RESULTS

Binding of DNSA by Carbonic Anhydrase—When DNSA was added to a solution of enzyme, the protein fluorescence excited at 280 mμ was quenched. The stoichiometry of the interaction of carbonic anhydrase with DNSA was studied by titration of a 10^{-5} M solution of protein with the sulfonamide, and the results are shown in Fig. 1. The quenching curve indicates that only 1 mole of ligand is bound per mole of protein. Since the extinction coefficients of the sulfonamide and the protein are known with some certainty (15, 18), the quenching curve can be used to calculate the molecular weight of the protein. With a solution of a weighed amount of carbonic anhydrase, a value of 29,500 was obtained, in good agreement with the value obtained by Nyman and Lindskog (15) from amino acid composition.

The binding of DNSA by carbonic anhydrase is accompanied by enhancement of ligand fluorescence, which again is consistent with the binding of 1 mole of sulfonamide (Fig. 1). Although it could be argued that additional DNSA might be bound without

DNSA; ANS, 8-anilinonaphthalene-1-sulfonic acid; BSA(DNSA), a complex of bovine serum albumin containing adsorbed DNSA; DNS-CA, a conjugate produced by reaction of carbonic anhydrase with DNS-chloride. Unless otherwise specified, the term carbonic anhydrase used in this paper refers to bovine erythrocyte carbonic anhydrase B.

[2] Quantum yield was determined by comparison of the integrated corrected emission spectrum of a standard with that of the unknown. If different excitation wave lengths were used for standard and unknown, adjustments were made for the differences in lamp intensity at those wave lengths (23). We have used 348 mμ excitation for the standard, quinine in 0.1 N H_2SO_4, and assumed its quantum yield to be 0.54. This value is based on the following data: Melhuish (24) found a quantum yield of 0.55 with 366 mμ excitation, but recent evidence shows that the quantum yield of quinine is 9% lower when excited at 348 mμ (25). If Melhuish's value is correct at 366 mμ, the quantum yield should be 0.50 with excitation at 348 mμ. However, Eastman (26) has recently obtained a quantum yield of 0.58 for quinine for excitation at 350 and 250 mμ. The value of 0.54 is an average of values based on the work of Melhuish and of Eastman.

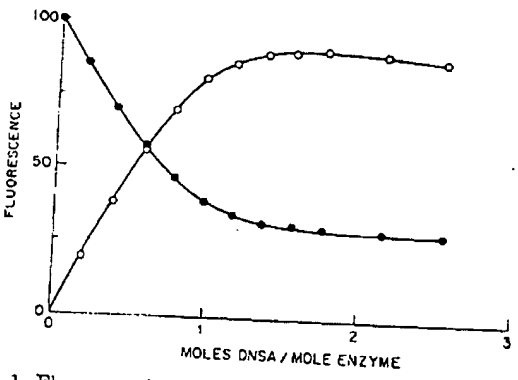

FIG. 1. Fluorometric titration of carbonic anhydrase at high concentration (8.2×10^{-6} M) with DNSA. The intensity of tryptophan fluorescence (●——●) was followed by excitation at 280 mμ with the emission monochromator set at 336 mμ; the corresponding wave lengths for ligand fluorescence (○——○) were 320 and 470 mμ. The emission of free DNSA at 470 mμ was negligible. Band widths of excitation and emission were 12 mμ.

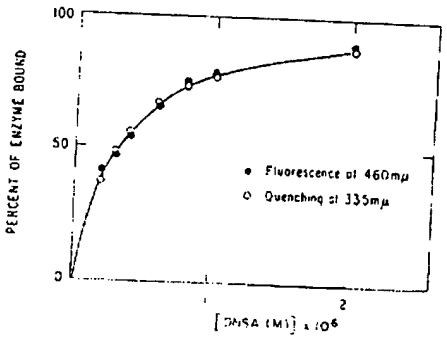

FIG. 2. Binding of DNSA to carbonic anhydrase in dilute solution, measured by quenching of protein fluorescence and enhancement of ligand fluorescence, with the emission wave lengths shown. The protein concentration was 2.1×10^{-7} M. The fraction of enzyme bound was assumed equal to the fraction of the maximum change of DNSA fluorescence enhancement or tryptophan fluorescence quenching as determined in experiments similar to that of Fig. 1.

fluorescence enhancement, this possibility is quite unlikely since one would then expect further quenching of protein fluorescence. The binding data of Fig. 1 could be used not only to show the stoichiometry but also to obtain the dissociation constant, K_D. However, K_D was obtained with greater precision by titrations at low enzyme concentration, as shown in Fig. 2. K_D was found to be 2.4 ($\pm$0.1) $\times 10^{-7}$ M. The experiment of Fig. 2 illustrates a point which will be expanded on in another communication;[3] namely, that fluorescence quenching is a very sensitive method of obtaining the binding constants of certain aromatic sulfonamides by carbonic anhydrase and permits titrations of solutions containing 10^{-7} to 10^{-6} M protein.

The 1:1 binding stoichiometry was also confirmed by another method. Ethoxzolamide, a highly potent inhibitor, has been found[3] by fluorescence quenching data to form a 1:1 complex with carbonic anhydrase with a K_D of 2.5×10^{-10}. Ethoxzolamide has negligible visible fluorescence; when it was added to solutions containing both carbonic anhydrase and DNSA, the enhancement of DNSA emission was blocked when equivalent amounts of enzyme and ethoxzolamide were present. The results of such a competition experiment are given in Fig. 3. Since ethoxzolamide is used clinically as a carbonic anhydrase inhibitor, the data provide evidence that the DNSA site is the binding site for such sulfonamide inhibitors.

Energy Transfer from Protein to DNSA—The quenching of carbonic anhydrase fluorescence by DNSA, like that observed in other protein interactions (30-32), is due to energy transfer from excited state tryptophanyl residues to bound ligand. A general prerequisite for energy transfer to occur by the Förster mechanism (33), involving induced dipole resonance, is that the emission band of the donor overlap the absorption band of the acceptor. Fig. 4 shows that this prerequisite is met in the system CA(DNSA), since the protein fluorescence band (λ_{max} 336 mμ) strongly overlaps the DNSA absorption band. The probability of energy transfer between two molecules can be expressed in terms of the critical transfer distance, R_0, for which resonance transfer is 50% complete by means of the equation (34, 35)

$$R_0 = \sqrt[6]{\frac{1.66 \times 10^{-25} \times \tau J_\nu}{n^2 \times \bar{\nu}_0^2}} \quad (1)$$

where τ is the donor fluorescence decay time, $\bar{\nu}_0$ the mean of the peak positions (in wave numbers) of the donor emission and lowest energy absorption bands, J_ν the overlap integral, and n the refractive index. Stryer (36) has applied the equation to proteins bearing DNS-groups and has obtained R_0 = 21 A for transfer from tryptophan to dye.

We have recalculated R_0 from Equation 1 from the spectral data of Fig. 4 for CA(DNSA), since it cannot be assumed that all DNS-protein systems have the same spectra. To obtain $\bar{\nu}_0$ we redrew the data of Fig. 4 in terms of the wave number scale, taking care to multiply the corrected emission spectrum of carbonic anhydrase by λ^2 according to Lippert *et al.* (37) in order to display the curve as relative quanta ($(dQ/d\nu)$ with respect to ν). By assuming that n = 1.6, and by using τ = 2.6 nsec (directly measured), we obtained $J_\nu = 6.01 \times 10^{10}$ cm^3 mM^{-2}, $\bar{\nu}_0 = 32.8 \times 10^3$ cm^{-1}, and R_0 = 21.3 A. The good correspondence of R_0 with that calculated by Stryer (36) for other proteins shows that the high quenching efficiency of DNSA on carbonic anhydrase is not due to any unusual spectral features such as an increase in oscillator strength or overlap integral; rather, the observations

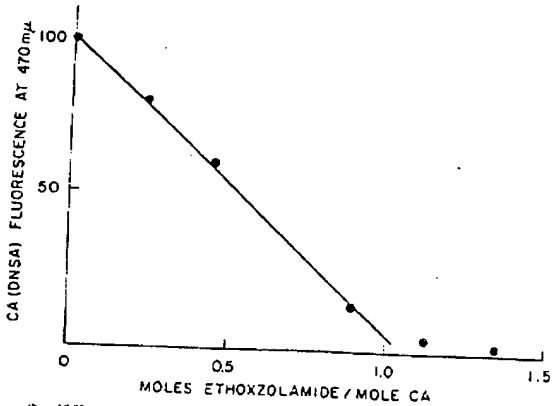

FIG. 3. Effect of ethoxzolamide on the visible fluorescence of CA(DNSA). Small aliquots of 10^{-3} M ethoxzolamide were added to a solution containing 5.6×10^{-7} M carbonic anhydrase and 3×10^{-6} M DNSA. Fluorescence was observed at 470 mμ; excitation, at 320 mμ.

[3] J. C. Kernohan, manuscript in preparation.

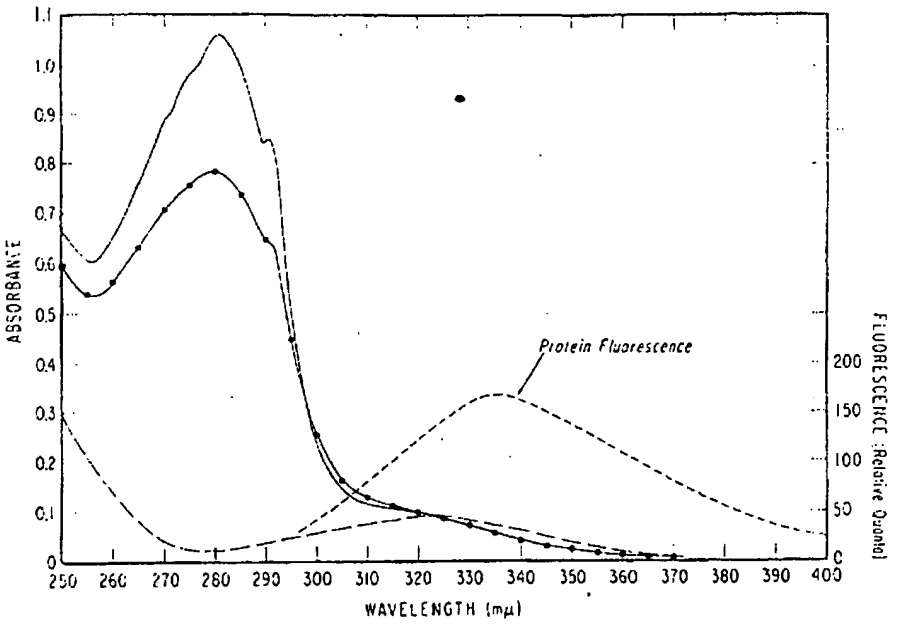

FIG. 4. Spectra of carbonic anhydrase and DNSA. Absorption spectra are shown for 2.0×10^{-5} M DNSA (– – –) and for a solution containing 1.94×10^{-5} M carbonic anhydrase and 2.0×10^{-5} M DNSA (——). Under these conditions, 91% of the enzyme is in the form of CA(DNSA). The corrected excitation spectrum of CA(DNSA) (●– – ●) was obtained by varying the exciting wave length while monitoring fluorescence at 468 mμ. The solution contained 4×10^{-6} M carbonic anhydrase and 10^{-5} M DNSA; fluorescence of free DNSA or free enzyme at 468 mμ was negligible. The excitation band width, 5 mμ, was much larger than that used in the Cary spectrophotometer for the absorption spectrum; hence, although the absorption and excitation spectra for CA(DNSA) are normalized at long wave length and are seen to match in that region, the difference in band widths may account for the mismatch near 300 mμ, where the absorption is changing rapidly. The corrected emission spectrum of native carbonic anhydrase, 3×10^{-5} M (-----), shows overlap with the absorption due to the DNS-group.

on quenching must be explained by particularly favorable spatial or geometrical factors, or both.

We recognize, however, that the transfer distance, R_0, calculated in the above manner is an approximation, since several assumptions must be made in order to use Equation 1 for protein-ligand systems. For instance, it is assumed that the orientations of the tryptophans of the protein are random with respect to the DNSA, that the various tryptophans all have the same emission spectra and lifetime, and that the refractive index in the protein matrix is 1.6. Nonetheless, because the transfer distance R_0 is proportional to the sixth root of the ratio $J_\nu/n^2\nu_0^2$, a 100% error in that quantity would result in only a 10% error in R_0.

The binding of only 1 mole of DNSA to carbonic anhydrase results in 73% quenching of protein fluorescence (Fig. 1). Because it is known that bovine carbonic anhydrase has 7 tryptophan residues (15), it seemed possible that quenching might involve certain residues more than others and therefore result in a change in the ultraviolet emission spectrum. However, with 290 mμ excitation, the emission spectra of carbonic anhydrase and of CA(DNSA) had the same shape and position. Thus, we cannot from this result distinguish between the following two possibilities: (*a*) the emission from the fluorescent tryptophans is 73% quenched by DNSA, or (*b*) the fluorescence of certain tryptophans is 100% quenched while that of other residues is quenched to a lesser degree, yet the resultant protein emission spectrum is unchanged since all the emitting residues have the same spectra.

More information on possible differences between the tryptophan residues is, however, obtained from the following considerations. Although the degree of quenching is related to the energy transfer process, the true efficiency of tryptophan-DNSA energy transfer cannot be calculated from quenching data alone, for the following reason. The quantum yield of tryptophan fluorescence in carbonic anhydrase is only 0.11 (Table I)[4]; thus 89% of the absorbed photons are lost by radiationless pathways. Since the quenching data concern only those 11% of the photons which are ordinarily emitted, one must turn to a comparison of the absorption and corrected excitation spectra of CA(DNSA) to determine the percentage of photons transferred to DNSA. Such spectra are shown in Fig. 4. The absorption spectrum for the solution containing DNSA and carbonic anhydrase is essentially that of CA(DNSA), since, under the conditions used, only 9% of the enzyme present was not in complex with the sulfonamide. Suitable corrections in the quantum yield determinations were applied for this amount of free enzyme. The excitation spectrum of CA(DNSA) is of interest because of the intense 280 mμ peak corresponding to the long wave length protein absorption band, whereas the spectrum of DNSA itself has a minimum in

[4] It should be noted that the fluorescence quantum yield of protein fluorescence was obtained with the quinine standard rather than with a tryptophan standard, which is often used. The quantum yield of tryptophan in water has been reported to be 0.20 (38), but experiments with various spectrofluorometers and with quinine as a standard have shown tryptophan to have a quantum yield of 0.13 in water (R. F. Chen and R. Perlman, to be published). The quantum yield of carbonic anhydrase is thus only slightly less than that of tryptophan which is free in aqueous solution.

is region. Above 300 mμ, the absorption and excitation spec- coincide and the quantum yield is 0.84. In the region where ht is absorbed by the protein, the fluorescence quantum yield lower. After correcting for the amount of light directly ab- bed by bound DNSA, we found that the quantum yields of the sible fluorescence excited by photons absorbed at 280 mμ and 0 mμ by the protein were 0.68 and 0.71, respectively. The ference in yields at these two wave lengths merely reflects the wer degree of energy transfer from tyrosine residues (36), which ntribute to absorption at 280 mμ much more than at 290 mμ. rom the result at 290 mμ, it is evident that the quanta absorbed tryptophan residues are transferred to DNSA with a yield of 5% (0.71/0.84 × 100). Also, since the quantum yield of tryp- phan fluorescence in the complex if 0.04 (quantum yield data re summarized in Table I), we can account for 89% of the hotons absorbed by tryptophan. The remaining 11% pre- mably suffer radiationless internal conversion directly from cited state tryptophans. The high degree of energy transfer eans that DNSA very effectively scavenges those photons which, the free enzyme, would have been lost by radiationless modes. or this reason, although the lifetime of the free enzyme fluores- nce is found to be only 2.6 nsec, the energy transfer process ust be considerably faster than even that. The rapidity of ergy transfer was confirmed by measuring the fluorescence cay time of CA(DNSA) by excitation with a deuterium flash mp in our lifetime apparatus. Using an ultraviolet inter- rence filter (Optics Technology, Palo Alto) to isolate a narrow nd centered at 290 mμ, we obtained τ = 21.8 nsec, which is not ignificantly different from τ = 22.1 nsec obtained by exciting rectly into the DNSA absorption band with the nitrogen sh lamp. Had the excited state of tryptophan persisted for, y, 2 nsec before transfer of energy, a larger value of τ would ave been detected.

It should be pointed out that the over-all energy transfer fficiency (85%) from tryptophan to DNSA is higher than the egree of quenching of tryptophan fluorescence (73%), which also due to energy transfer. The simplest explanation for this difference is that the quantum efficiencies of the various trypto- phan residues are not identical and that DNSA is situated in such a manner that energy transfer is more probable from those tryptophans which are relatively less fluorescent. In a simple model system in which all donor groups were identical, Latt, Cheung, and Blout (39) showed that the degree of fluorescence quenching by an energy acceptor group was equal to the degree of energy transfer. With regard to the possibility that the tryptophans in a protein may be heterogeneous, it is known that the fluorescence due to tryptophan varies in yield in different proteins and is markedly affected by the environment (40). An alternative explanation which cannot be wholly discounted at present is that the binding of DNSA causes a conformational change which influences the quantum yield of tryptophan emis- sion.

Nature of Binding Site—The site occupied by DNSA appears to be quite specific for unsubstituted sulfonamides. DNSA can be displaced from carbonic anhydrase by other sulfonamides such as ethoxzolamide, benzenesulfonamide, and acetazolamide (1,3,4-thiazole-2-acetamido-5-sulfonamide).[3] On the other hand, attempts to detect complexes of this enzyme with ANS, DNS-glycine, DNS-L-glutamic acid, DNS-L-proline, and DNS-L-tryptophan gave negative results in terms of protein fluores- cence quenching, ligand fluorescence enhancement, or increase in polarization of visible fluorescence. These compounds are known to form highly fluorescent complexes with proteins such as serum albumin, apomyoglobin, and apohemoglobin (8, 11, 14); however, they lack the basic primary sulfonamide structure needed to bind to carbonic anhydrase.

TABLE I

Quantum yields and decay times

The quantum yields refer only to the emission band cited in the preceding column. The total quantum yield of CA(DNSA) excited at 290 mμ would thus be 0.71 + 0.04 = 0.75. Solvent for protein solutions was 0.02 M potassium phosphate buffer, pH 7.4; DNSA was dissolved in dilute HCl and the solutions were brought to the desired pH by addition of KOH. The complex BSA(DNSA) was prepared by adding DNSA to a final concentration of 2×10^{-6} to 10^{-4} M bovine serum albumin.

Compound	Exciting wave length	λ_{max} of emission	Quantum yield	τ^a
	mμ	mμ		nsec
Carbonic anhydrase	290	336	0.11	2.6
CA(DNSA)	290	336	0.04	
CA(DNSA)	290	468	0.71	21.8
CA(DNSA)	320	468	0.84	22.1
DNSA, pH 7.4	320	580	0.055	3.9
DNSA, pH 12	310	538	0.085	4.0
DNS-CA	335	533	0.12	12.2
DNS-CA	290	336	0.090	
BSA(DNSA)	340	500	0.64	22.0

[a] Fluorescence decay times were measured in the nanosecond flash fluorometer with suitable Corning or interference filters to give excitation at the wave lengths listed, which were used for the determination of quantum yields.

The changes in DNSA emission properties upon binding to the enzyme are shown in Fig. 5 summarized in Table I. At pH 7.4, free DNSA has a weak fluorescence with a peak at 580 mμ and a quantum yield of 0.055. In complex with carbonic anhydrase, DNSA emits maximally at 468 mμ with a yield of 0.84. The 112 mμ blue shift and the 15-fold quantum yield increase require comment.

In a recent study of the DNS-amino acids, it was shown that their emission spectra have peaks near 580 mμ in water and move toward shorter wave lengths in hydrophobic organic solvents (14). In dioxane, for instance, it was found that DNS-tryptophan had a peak at 500 mμ. Upon binding of DNS-amino acids such as DNS-L-proline to serum albumin, the peak of the spectrum of the visible fluorescence varied with the species of albumin tested and ranged from 486 to 502 mμ. The effects of solvents and protein solutions on the fluorescence of DNS-amino acids were interpreted in terms of a high sensitivity of the compounds to the dielectric constant of the environment (14). DNSA has the same chromophoric group as the DNS-amino acids, and, by spectral measurements in dioxane-water mixtures, we have found that DNSA shows similar solvent shifts in its emission. Furthermore, DNSA binds to bovine serum albumin, and the fluorescence spectrum is given in Fig. 6. The quantum yield of the fluorescence excited at 340 mμ is 0.64, and the peak is at 500 mμ. It is clear, then, that the hydrophobic nature of the binding site can account for part of the blue shift exhibited by CA(DNSA). However, the emission of CA(DNSA) is considerably bluer than that of the corresponding bovine serum

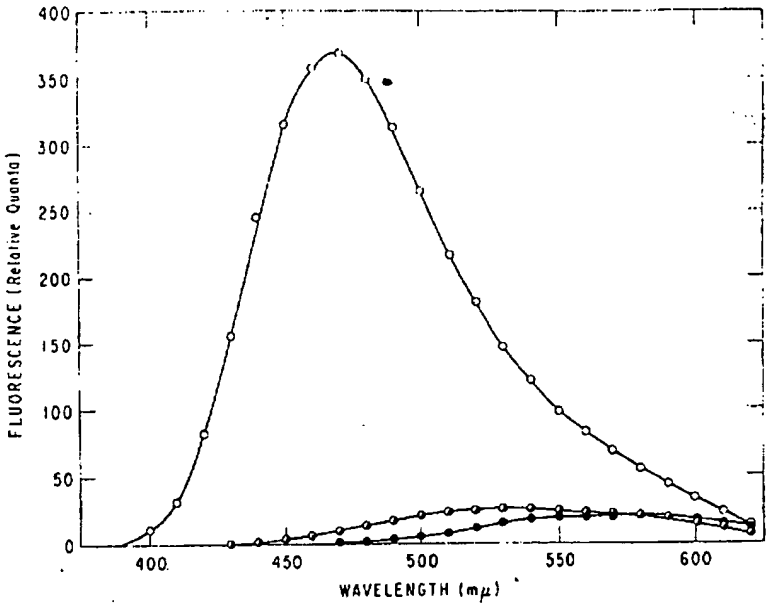

FIG. 5. Corrected emission spectra of DNSA and CA(DNSA). The curves have been adjusted so that the areas underneath the lines are proportional to the quantum yields (which are listed in Table I). The spectra are of CA(DNSA) (O——O) excited at 310 mμ, and of 1.0×10^{-6} M DNSA in 0.02 M potassium phosphate buffer, pH 7.4 (●——●), and in 0.01 M KOH (◑----◑). The solution used for the CA(DNSA) fluorescence was the same as that used for the absorption spectrum in Fig. 4.

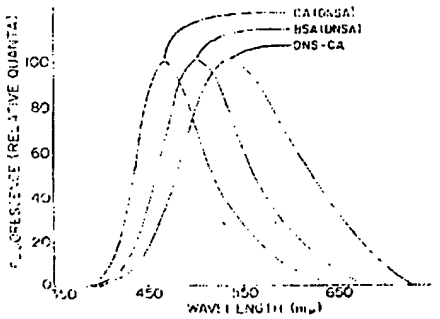

FIG. 6. Emission spectra of DNS-containing proteins. The spectrum of CA(DNSA) is replotted from Fig. 5 for comparison. The curves are normalized to the same height, although the quantum yields (Table I) are different. The emission of BSA(DNSA) was obtained by 340 mμ excitation of a solution described in Table 1. The spectrum of DNS-CA, 3×10^{-6} M, was obtained by 335 mμ excitation. Band width of emission monochromator, 12 mμ.

albumin complex, in spite of the fact that albumin has a very hydrophobic binding site (10, 41). Therefore, yet another factor must be involved in determining the visible fluorescence spectrum of CA(DNSA).

We suggest that such a factor may be the loss of a proton from DNSA, i.e., the process $ArSO_2NH_2 \rightleftharpoons ArSO_2NH^- + H^+$. Some of the evidence from other work that sulfonamides are bound to carbonic anhydrases in the form $ArSO_2NH^-$ is considered under "Discussion." In addition, the following spectral characteristics of DNSA are consistent with loss of a proton upon binding to the enzyme. It is evident from Fig. 4 that the absorption spectrum of DNSA is markedly blue-shifted on binding to carbonic anhydrase. A difference spectrum was obtained with 6×10^{-5} M carbonic anhydrase in the reference cell and the same solution plus 5×10^{-6} M DNSA in the sample cell; the results showed bound DNSA to have an absorption maximum at 309 to 310 mμ with $\epsilon = 4.50 \times 10^3$ M^{-1} cm^{-1}. In contrast, free DNSA has a peak at 323 mμ (Fig. 4). A blue shift in the absorption spectrum of DNSA can also be induced by raising the pH, and we found that the spectrum of this compound in 0.01 M KOH had a peak at 310 mμ with $\epsilon = 5.59 \times 10^3$ M^{-1} cm^{-1}. The blue shift in absorption at high pH, which was reversible, seems clearly to be due to ionization of the sulfonamide moiety. The process is accompanied by a blue shift in the fluorescence emission spectrum, with the peak changed from 580 mμ to 540 mμ (Fig. 5) and the quantum yield rising from 0.055 to 0.085. It might be expected, therefore, that ionization of the sulfonamide group on binding to carbonic anhydrase would involve a fluorescence blue shift of similar magnitude. Loss of a proton from DNSA on binding to the enzyme could thus account for the difference in position between the emission spectra of CA(DNSA) and BSA(DNSA) shown in Fig. 6.

The absorption blue shift shown by DNSA in alkali allowed us to study the ionization in more detail by titrating and monitoring the appearance of ionized DNSA by the increase in absorption at 305 mμ (Fig. 7). The pK_a of the $—SO_2NH_2$ group was found to be 9.79. Some compounds are stronger acids in their excited states; the subject of *photo*ionization or ejection of protons from excited states has been reviewed recently by Wehry and Rogers (42). Since Lagunoff and Ottolenghi (18) have shown that the protonated dimethylamino group of 5-dimethylaminonaphthalene-1-sulfonic acid at low pH was a much stronger acid in the excited singlet state, we examined the question whether the sulfonamide group of excited DNSA ionized more readily than the ground state. Fluorometric titration of DNSA performed by monitoring the appearance of ionized sulfonamide at 500 mμ showed exactly the same pK_a as spectrophotometric titration (Fig. 7). One can conclude, therefore, that if ejection of a proton from excited state DNSA occurs the process must be much

slower then fluorescence, for which the decay time was measured to be about 4 nsec (Table I), and would not be detectable experimentally.

An additional implication of the blue shifts exhibited by DNSA in alkali has been suggested to us by a referee. In compounds such as certain naphthols, in which the excited state is a much stronger acid than the ground state, loss of a proton is associated with large spectral red shifts, as can be predicted from thermodynamic considerations (42). The spectra of DNSA shift in the opposite direction, a finding which suggests that differences in ground and excited state acidities of the $-SO_2NH_2$ group are, in fact, not great.

The ionization of the sulfonamide group does not seem to be incompatible with the emission blue shift due to the transfer of DNSA from aqueous to hydrophobic environments. This was confirmed by investigation of the emission spectra of DNSA in solutions containing different proportions of dioxane and aqueous KOH. The effects of solvent and ionization of the sulfonamide group on the emission spectrum appear to be more or less additive. For instance, it was found that in 90% dioxane-10% 1 M KOH the emission maximum of DNSA was at 490 mμ, whereas in 90% dioxane-10% 0.1 M potassium phosphate, pH 7.4, the peak was at 502 mμ.

Inhibition of Carbonic Anhydrase by DNSA—In order to test the effect of DNSA on the activity of carbonic anhydrase, we employed the esterase assay with the substrate, *p*-nitrophenyl acetate (43). In the absence of inhibitor, linear Lineweaver-Burk plots were obtained, with $K_m = 4 \times 10^{-3}$ M and $V_{max} = 2.5 \times 10^3$ moles per min per mole of enzyme in 0.02 M potassium phosphate buffer, pH 7.4. DNSA proved to be a powerful inhibitor. The inhibitor constant, K_I, as defined by Armstrong *et al.* (20), was obtained by their method, and found to be 4×10^{-7} M. The inhibition was apparently noncompetitive, because V_{max} was decreased at all inhibitor concentrations used.

It should be recalled that, although the inhibition of DNSA and other sulfonamides have been found by classical techniques to be noncompetitive (20, 44, 45), it has been pointed out that the inhibitors may still actually bind to the catalytic site (7). The apparently noncompetitive nature of the inhibition could be due to a relatively slow rate of release of inhibitor. One of our observations that supports this possibility is that the visible fluorescence of the CA(DNSA) complex disappears only slowly when ethoxzolamide is added to the solution. The kinetics of the displacement of DNSA is currently under study, and preliminary results suggest that the obliteration of CA(DNSA) fluorescence by ethoxzolamide has a half-time on the order of 3 to 4 sec.

Rotational Relaxation Time of Carbonic Anhydrase—The method used by Weber (46) to obtain rotational relaxation times utilizes proteins covalently labeled with fluorescent dyes. With the explicit assumptions that (*a*) the labeling is random and (*b*) the macromolecule rotates as a rigid entity, the mean harmonic rotational relaxation time, ρ_h, can be obtained by measurements of the fluorescence polarization at different temperatures and viscosities. The Perrin-Weber equation (46) is

$$\frac{1}{P} - \frac{1}{3} = \left(\frac{1}{P_0} - \frac{1}{3}\right)\left(1 + \frac{3\tau}{\rho_h}\right) \qquad (2)$$

where P and P_0 are the fluorescence polarizations with linearly polarized exciting light in media of finite and infinite viscosities, respectively, and τ is the fluorescence decay time. The relaxation time, ρ_h, obtained this way is often compared with the relaxation time, ρ_0, of the anhydrous sphere of equivalent molecular weight obtained from (46)

$$\rho_0 = \frac{3\eta V}{RT} \qquad (3)$$

where η is the viscosity, V the molecular volume, R the gas constant, and T the absolute temperature. Equation 2 is used in conjunction with measurements of P at different values of T/η to give ρ_h; plots of $1/P$ with respect to T/η are extrapolated to infinite viscosity to give P_0, and τ is independently measured.

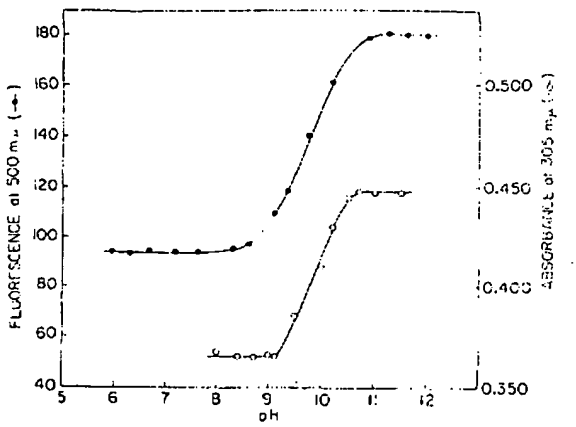

FIG. 7. Titration of DNSA. DNSA, originally dissolved in 0.001 M HCl, was titrated by addition of KOH, and the optical parameters were followed by absorption at 305 mμ in a 10^{-4} M solution, or by the increase in fluorescence at 500 mμ excited at 310 mμ in a 10^{-6} M solution.

For the CA(DNSA) complex, a fluorescence decay time at 25° of 22.1 nsec was obtained, and measurements of the polarization of fluorescence as a function of temperature yielded the Perrin-Weber plot shown in Fig. 8. At 25°, the relaxation time is thus 28.9 nsec. From Equation 3, $\rho_0 = 24.3$ nsec, with the assumption that the molecular weight is 30,000 and the partial specific volume is 0.73, so that the ratio ρ_h/ρ_0 is 1.19. For most proteins rotating as rigid ellipsoids, the relaxation time ratio ρ_h/ρ_0 has been found to be between about 1.7 and 2.0 (47). The low ρ_h/ρ_0 ratio observed here for CA(DNSA) suggests that the molecule has a low degree of asymmetry, and there is some indirect evidence for this. The measurements of intrinsic viscosity by Armstrong *et al.* (20) on human carbonic anhydrase B suggested that the protein must be nearly spherical. Work of Nyman and Lindskog (15, 48) shows that there are a number of similarities between bovine carbonic anhydrase B and the human enzymes; these similarities include aspects of the amino acid compositions, molecular weights, sedimentation coefficients, and affinities for zinc. Similarities in molecular shape might also be expected.

A possible criticism of the use of the fluorescence depolarization method in the case of a protein complex containing a single fluorescent group is that the labeling can in no sense be considered "random," as was assumed in the derivation of Equation 2 (46). When a single moiety is bound to a macromolecule, it is possible that the results will be influenced by preferential orientation of the electronic oscillators of the ligand with respect to the axes of the protein ellipsoid (49). While such a criticism might be applicable to relaxation time studies already reported with ANS bound to bovine serum albumin or to apomyoglobin and apo-

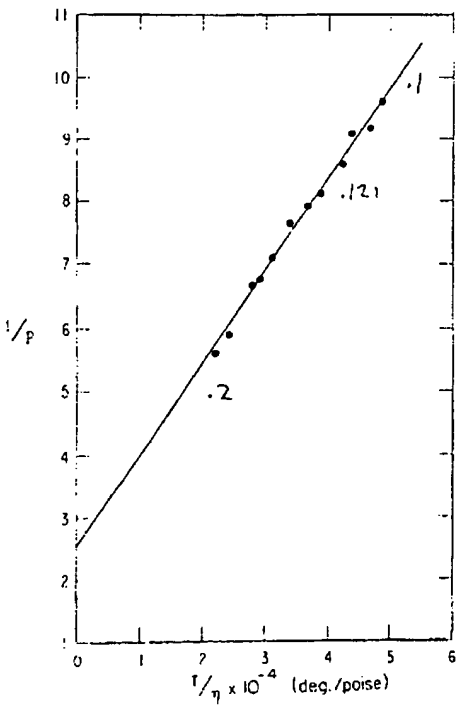

FIG. 8. Perrin-Weber plot for the depolarization of CA(DNSA) fluorescence. A solution of 4.4×10^{-6} M carbonic anhydrase containing 6×10^{-6} M DNSA in 0.02 M potassium phosphate buffer was excited with polarized light at 320 mμ, and the emitted light was analyzed with the monochromator set at 470 mμ. The data were analyzed by the C-E-I-R, Inc., on site computer service to determine the slope and intercept of the line defined by the points according to a mean least squares approximation.

hemoglobin (8, 9), the objection will be only minor in the case of bovine carbonic anhydrase, if, like the human enzyme (20), its shape is nearly spherical. Furthermore, the relaxation time was similar to those obtained with a conjugate prepared by reaction of carbonic anhydrase with DNS-chloride, as is discussed below.

Properties of DNS-CA Conjugate—It seemed desirable to compare the properties of CA(DNSA) with those of a conjugate of the enzyme which contained the same number of DNS-groups. The conditions found to yield such a conjugate are as follows. To 1 ml of 0.0 M $NaHCO_3$ containing 10 mg of carbonic anhydrase was added 0.1 ml of acetone containing 0.2 mg of DNS-chloride, and the mixture was stirred in an ice bath for 4 hours. The mixture was then dialyzed for 48 hours at 2° against 1 liter of 0.002 M potassium phosphate buffer, pH 7.0, containing 3 g of activated charcoal as a slurry (Darco). The dialysate was changed twice in this time. The dialyzed protein was free of unconjugated dye as determined by paper chromatography (50). The average degree of labeling was determined spectrophotometrically (17) and found to be 1.1 moles of DNS- per mole of protein; the absorption spectrum of the complex is shown in Fig. 9.

There were numerous differences between the conjugate and the complex, CA(DNSA). First, the absorption spectrum has a maximum at 335 mμ for the DNS- band (Fig. 9); this is to be compared with a peak at 309 to 310 mμ obtained from the difference spectrum of CA(DNSA) minus enzyme alone (*cf.* Fig. 4). Second, energy transfer from tryptophan to DNS- in the conjugate is only 10% efficient, as is shown by calculations based on the excitation and absorption spectra of Fig. 9. The data of Figs. 4 and 9 were used to calculate the transfer distance R_0 for the conjugate, and R_0 was found to be 22.8 A. The transfer distances for conjugate and complex are thus similar; the small difference was due to greater spectral overlap (higher J_ν) in the case of the conjugate. Third, the quantum yield of tryptophan fluorescence in the conjugate was 0.09, compared with 0.11 in the unlabeled protein. In the conjugate, therefore, quenching by DNS- occurs to the extent of only 20% compared with 73% in CA(DNSA). Fourth, the fluorescence characteristics of the DNS- conjugate were quite dissimilar to those described for CA(DNSA). Instead of a blue fluorescence, the emission of conjugate appeared green under a ultraviolet light, and the corrected emission spectrum had a maximum at 533 mμ (Fig. 6). The quantum yield was 0.12 (Table I), and the fluorescence decay time was 12.2 nsec in 0.02 M potassium phosphate buffer, pH 7.4. Fifth, although our data indicated that the enzyme containing bound DNSA is inactive, we found the DNS-conjugate to be 100% as active as the unlabeled enzyme in the esterase assay.

Calculation of Mean Effective Transfer Distance—The energy transfer data obtained with CA(DNSA) and DNS-CA permit calculation of a mean effective distance, R, between donor and acceptor groups. From transfer theory (34, 51),

$$1 - X = \frac{1}{(R_0/R)^6 + 1} \qquad (4)$$

where X is the fraction of the absorbed photons which are transferred. In CA(DNSA), $X = 0.85$, $R_0 = 21.3$ A, and $R = 16.0$ A. For the conjugate, DNS-CA, $X = 0.10$, $R_0 = 22.8$ A, and $R = 32.9$ A. On the average, therefore, the tryptophans in the conjugate are about twice as far from the DNS- group as they are in the DNSA complex. An anhydrous sphere of 30,000 molecular weight and a specific volume of 0.73 would have a

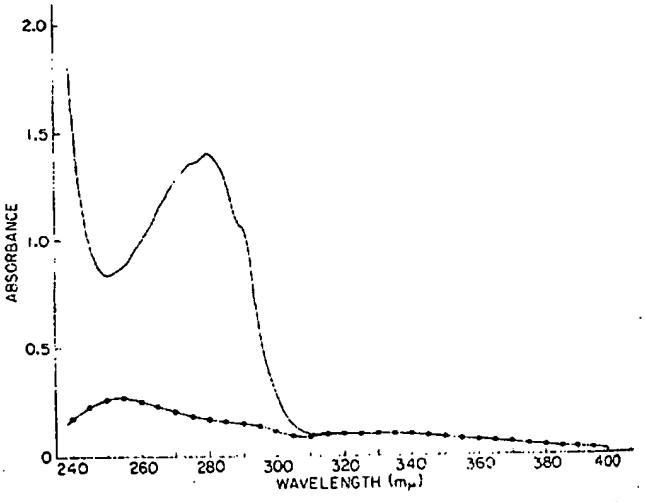

FIG. 9. Energy transfer in a DNS conjugate of carbonic anhydrase. The absorption spectrum (——) of 2.4×10^{-4} M DNS-CA and the excitation spectrum of the same solution diluted 10-fold (●——●) have been normalized at long wave lengths, where the two curves coincide. The excitation spectrum was obtained with an 8-mμ excitation band width, the emission being observed at 540 mμ.

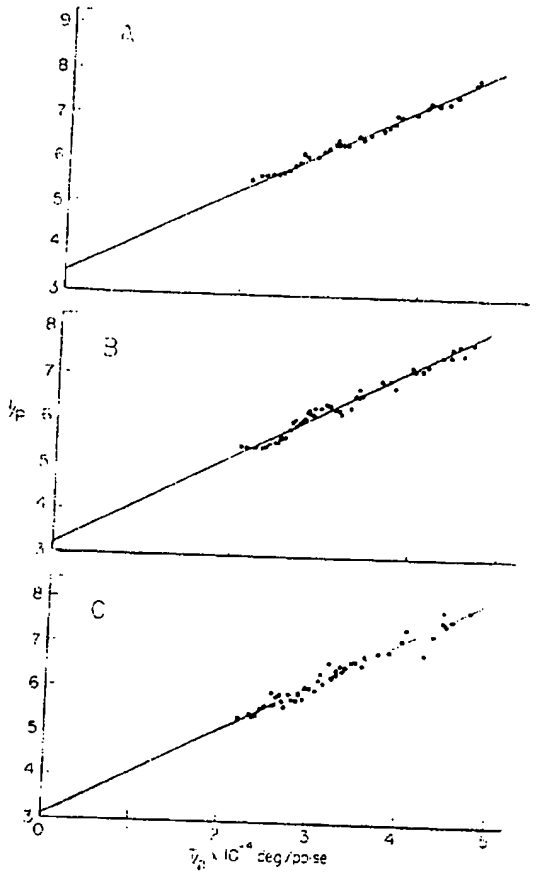

FIG. 10. Perrin-Weber plots for DNS-CA. 10^{-5} M solutions of DNS-CA were examined in 0.02 M potassium phosphate buffer, pH 7.4 (*A*), the same buffer plus 10^{-4} M ethoxzolamide (*B*), and 0.1 M $NaHCO_3$ (*C*). The data were analyzed as in Fig. 8. Exciting wave length, 335 mμ; emission was observed at 540 mμ.

diameter of 51 A. The small value of R for CA(DNSA) again suggests segregation of the tryptophans in the vicinity of the DNSA site.

Except to point out the large difference in R for the two preparations, too much emphasis should not be placed on the use of Equation 4 for a system as complex as a protein. The equation is subject to the same reservations pointed out previously for Equation 1. In view of the evidence presented above for the heterogeneity of the donor groups (tryptophans), the concept that a single R_0 governs each tryptophan-DNS- pair is, to some extent, a fiction.

Fluorescence Depolarization in DNS-Conjugate—The polarization of fluorescence of the conjugate was measured as a function of increasing temperature, and the resulting Perrin-Weber plots are shown in Fig. 10. At 25° with solutions containing 10^{-5} M protein, the following results were obtained: Fig. 10*A*, in 0.02 M potassium phosphate, pH 7.4, τ = 12.2 nsec and ρ_h = 34.9 nsec; *B*, in the same buffer plus 10^{-4} M ethoxzolamide, τ = 12.3 nsec and ρ_h = 31.9 nsec; *C*, in 0.1 M $NaHCO_3$, τ = 11.5 nsec and ρ_h = 29.0 nsec.

All the available evidence indicates that the DNS- label in the conjugate is in a different position on the protein than it is in CA(DNSA), but there is no information on whether the DNS-label of the conjugate is randomly placed or whether a single site is occupied. In any case, it is of interest that the relaxation times obtained with either DNS-CA or CA(DNSA) are similar. This fact indicates that the relaxation time of carbonic anhydrase is indeed about 30 nsec, that the asymmetry of the protein is not great enough to render objectionable the nonrandomness of fluorescent labeling, and that the fluorescent labels are probably tightly bound to the protein.

DISCUSSION

The present studies on the binding of fluorescent groups to bovine carbonic anhydrase B provide some direct spectral information bearing on the chemical nature of the sulfonamide-binding site. The very high quantum yield and the fluorescence spectrum of bound DNSA suggest that the site is shielded from solvent interactions and has a highly hydrophobic character. As pointed out above, the fact that activity studies with sulfonamides have given Lineweaver-Burk curves suggestive of noncompetitive inhibition does not rule out the possibility that the sulfonamides bind at the site required for the catalysis of CO_2 hydration. If the active site and the sulfonamide-binding site are indeed identical, we are left with the mild paradox that water reacts at or near a hydrophobic site.

The reason for the strong binding and inhibitory power of aromatic sulfonamides has been unclear for some time, since sulfonamides are only weak ligands for Zn^{++}. This study suggests that the sulfonamide-binding site consists of two sections, one of which promotes the dissociation of a proton from the $-SO_2NH_2$ group, and the other, a hydrophobic binding region which attracts the aromatic portion of the sulfonamide. Aside from the spectral evidence provided here for dissociation of the sulfonamide group, there is reasonably good kinetic evidence supporting such a scheme. Miller, Dessert, and Roblin (3) postulated that ionization of the sulfonamide group was necessary for attachment to carbonic anhydrases because the inhibitory power of a series of sulfonamides was related to the pK_a, compounds having more acidic $-SO_2NH_2$ groups being in general more potent inhibitors. More recently, the inhibition of bovine carbonic anhydrase by benzenesulfonamide was followed by stop-flow techniques as a function of pH. An inflection in the pH-activity curve in the region of the sulfonamide pK_a again indicated that dissociation of a proton from the inhibitor occurs on binding (4).

A hydrophobic character of the sulfonamide-binding site has not previously been found by chemical techniques. It is of interest, however, that the hydrophobic portion of the binding site is clearly not able by itself to cause significant binding of hydrophobic ligands such as ANS or the DNS-amino acids.

If one can assume that the aromatic sulfonamide inhibitors bind to carbonic anhydrase through two groups (via a hydrophobic bond involving the aromatic nucleus and also through a bond involving the ionized sulfonamide moiety), a ready explanation for the large association constants for these inhibitors is at hand. The inhibitors may be likened to bidentate ligands capable of forming chelates with transition metal ions. Schwarzenbach (52) pointed out that the formation constant for a chelate containing a bifunctional ligand is almost always much larger than the corresponding constant for the formation of a complex containing two monofunctional ligands. This "chelate effect" has been analyzed in terms of entropic and statistical factors favoring the stability of the chelate (52).

The energy transfer from tryptophan to DNSA is much more

efficient than has been observed hitherto in any DNS-containing protein system. For instance, Stryer (36) studied the energy transfer from tryptophan to DNS- in conjugates of chymotrypsinogen, a protein having a molecular weight of 24,000. When 5.5 DNS- groups were attached to the protein by reaction with DNS-chloride, 74% of the photons absorbed by tryptophan residues were transferred to the dye groups. These results are to be contrasted with the data here reported for the larger CA (DNSA) complex, in which energy transfer was 85%, with only 1 DNS- group. The critical transfer distance, R_0, for the tryptophan-DNS- pair calculated by Stryer (39) is about the same as that found here for either CA(DNSA) or the DNS-conjugate of the enzyme. Therefore, the much higher degree of energy transfer in CA(DNSA) must be due to nearness to or favorable orientation *vis à vis* the tryptophan residues, or both.

In this regard, we should point to some other evidence concerning the location of the tryptophans and the sulfonamide-binding site. Although much of this evidence was derived with human carbonic anhydrase C, that enzyme has many similarities (molecular weight, amino acid composition, zinc-binding affinity, activity, and so forth) with bovine carbonic anhydrase B. Optical perturbation experiments by Riddiford (53) on the human enzyme suggest that the tryptophans are buried in the interior of the molecule. Further, Urry and Eyring (54) suggest that in human carbonic anhydrase at least 2 tryptophans may be in fixed juxtaposition, from evidence obtained by optical rotatory dispersion in the ultraviolet region.

Very recently, Fridborg *et al.* (6) published the results of a study at 5.5-A resolution on the x-ray scattering of crystals of human carbonic anhydrase C containing the inhibitor acetoxymercurisulfanilamide. Even at this relatively low resolution, it was possible to make several observations. Zinc was found in the center of the molecule near the carboxyl terminus of the polypeptide chain. The inhibitor occupied part of a deep crevice containing the zinc and was bound to the metal ion through the sulfonamide group. The benzene ring of the inhibitor was seen to lie in a narrow slot. If this picture has validity also for bovine carbonic anhydrase B, the x-ray data would confirm the fluorescence evidence that the sulfonamide-binding site is well shielded and is in the interior of the protein.

The validity of Förster's theory of energy transfer has been tested and essentially confirmed by studies on models containing donor and acceptor groups in the same molecule (*e.g.* References 39, 55-57). Latt, Cheung, and Blout (39) studied energy transfer from the 1-naphthoyl group to anthracene-9-carbonyl in a rigid molecule and obtained rather direct evidence that the mutual orientation of the donor-acceptor pair affected transfer efficiency. Similar tests of Förster's theory have been impractical in proteins, but if further x-ray crystallographic data are forthcoming on carbonic anhydrases it may be possible to know the positions of the tryptophans with great accuracy and to correlate this information with energy transfer data such as those reported in this work.

Acknowledgment—Mrs. C. Scott provided expert technical assistance.

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